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In Vitro and *In Vivo* Activity of Omadacycline Against Two Biothreat Pathogens: *Bacillus anthracis* and *Yersinia pestis*

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ABSTRACT

Introduction: The *in vitro* activity and *in vivo* efficacy of omadacycline (OMC) were evaluated against the causative pathogen of anthrax and plague, *Bacillus anthracis* and *Yersinia pestis*, respectively.

Methods: Minimum inhibitory concentrations (MICs) of OMC were determined by microbroth dilution according to CLSI guidelines for 30 isolates each of *Yersinia pestis* and *Bacillus anthracis*. The *in vivo* efficacy of omadacycline was studied at a range of dosages in both a post exposure prophylaxis (PEP) murine model of anthrax and plague as well as in a delayed treatment model of inhalational anthrax.

Results: Omadacycline was active *in vitro* against *Y. pestis* ($MIC_{90=}$1 mcg/mL) and *B. anthracis* ($MIC_{90=}$0.06 mcg/mL). Omadacycline was less active *in vitro* than ciprofloxacin (CIP) against *Y. pestis* (CIP MIC_{90}=0.03 mcg/mL), but more potent *in vitro* against *B. anthracis* (CIP MIC_{90}=0.12 mcg/mL). In the mouse model of infection, the survival curves for all treatment cohorts differed significantly from the vehicle control (p=0.004). The median survival for the vehicle-treated controls was 6 days' post-challenge while all antibiotic-treated mice survived the entire study. Omadacycline treatment with 5, 10 or 20 mg/kg twice daily for 14 days had significant efficacy over the vehicle control in the treatment of aerosolized *B. anthracis*. Additionally, for post exposure prophylaxis treatment of mice infected with *Y. pestis*, the survival curves for omadacycline (40 mg/kg twice daily), ciprofloxacin, and doxycycline cohorts differed significantly from the vehicle control (p<0.0001).

Conclusions: Omadacycline is potent and demonstrates efficacy against both *B. anthracis* and *Y. pestis*. The well-characterized oral and IV pharmacokinetics, safety and tolerability, warrant further assessment of the potential utility of omadacycline in combating these serious biothreat organisms.

INTRODUCTION

In the past 15 to 20 years the threat of bioterrorism has increased as a result of increasing political and economic unrest in many parts of the world (Rai and Kaur, 2013; Weiss et al, 2015). The Centers for Disease Control (CDC) has classified bioterrorism agents into three categories based on their potential to cause severe disease that results in high rates of mortality, and according to how readily these agents can be disseminated in the general population (Rotz et al, 2002). Among the bioterrorism agents that pose the highest threat are *Bacillus anthracis* and *Yersinia pestis*, which are the causative pathogens for anthrax and plague, respectively. Current antibiotic treatment options against these Category A Biothreat pathogens are limited and the potential for engineered or evolved antibiotic resistance is high, thus, new therapeutic options are needed for prophylaxis and treatment of the diseases caused by these pathogens (Schweizer, 2012; Hatcher et al, 2015; Steed et al, 2015; Ingelsby et al, 1999; Ingelsby et al, 2000). Few new oral antibiotics are in development for the treatment of biothreat pathogens, and those older agents that have been approved are facing increasing resistance problems and could face engineered resistance.

As a class, tetracyclines have been used for over 60 years and have proven effective and well tolerated for the treatment of a variety of bacterial infections including those caused by many of the bacterial pathogens considered to be high priority biologic threats (Plague, Anthrax). However, reports of resistance to

tetracyclines, including doxycycline, and to fluoroquinolones and beta-lactams, have appeared in the literature, and these reports highlight the need for new treatment options for these biothreat agents (Schweizer, 2012). In addition, recent safety concerns for the fluoroquinolones potentially limits their utility (FDA Warning, 2016).

Omadacycline is a novel aminomethylcycline of the tetracycline family, designed to overcome mechanisms of resistance to the tetracycline class (Honeyman et al, 2015; Draper et al, 2014; Macone et al, 2014). The extensive preclinical and clinical development program for omadacycline is based on its demonstrated potent activity against key pathogens for serious community-acquired infections, including methicillin-resistant *Staphylococcus aureus*, multidrug resistant *Streptococcus pneumoniae*, Gram-negative aerobes, and atypical pathogens, and its lack of cross resistance to older generation tetracyclines and other antibiotic classes (Flamm et al, 1995a, b, c; Dubois et al, 2015; Kim et al, 2016). Omadacycline is currently in clinical development for acute bacterial skin and skin structure infection (ABSSSI) and community acquired bacterial pneumonia (CABP) as oral and intravenous (IV) monotherapy. Because of its broad spectrum *in vitro* activity, clinical profile, and oral bioavailability, omadacycline would be well suited for use in the treatment or post-exposure prophylaxis of infections of concern in both the biodefense and public health settings. This study evaluated the *in vitro* and in vivo activity of omadacycline against *B. anthracis* and *Y. pestis.*

METHODS

In Vitro Study

Minimum inhibitory concentrations (MICs) were determined by the microdilution method in 96-well plates according to Clinical and Laboratory Standards Institute (CLSI). Antibiotics were serially diluted two-fold in 50 µL of cation-adjusted Mueller-Hinton broth (CAMHB). The antibiotic ranges were 8 - 0.004 mcg/mL or 64 - 0.03 mcg/mL based on a final well volume of 100 µL after inoculation.

Bacterial inoculums were prepared by suspending colonies into CAMHB from 18-24 hours (*B. anthracis*) or 42-48 hours (*Y. pestis*) on Sheep Blood agar (SBA) plates that were incubated at 35ºC. p. Suspended cultures were diluted with CAMHB to a bacterial cell density of 10^5 CFU/mL adjusted based on OD600. To each well of the 96-well plate, 50 µL of dilutions was added for a final inoculum of ~5 x 10^4 CFU/well. Plates were incubated at 35ºC. MICs were determined visually at 18-24 hours (*B. anthracis*) or 42-48 hours (*Y. pestis*) and also by absorbance at 600 nm (SpectroMax M2, Molecular Devices). Thirty strains representing the genetic and geographic diversity of each bacterial species were used in these studies. Quality control of antibiotic stocks was established by using *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213.

In Vivo Studies

Preparation of B. anthracis and Y. pestis Strains

The United States Army Medical Research Institute of Infectious Diseases (USAMRIID) obtained the *B. anthracis* Ames strain from the United States Department of Agriculture, Ames Iowa. It was originally isolated in 1981 from a dead cow in Texas. Purified spore preparations of the organism are maintained at the Test Facility. *B. anthracis* Ames spores were prepared according to the method of Leighton and Doi (Leighton and Doi, 1971). The 50% lethal dose (LD_{50}) in mice was 3.4×10^4 colony-forming units (CFU) inhaled when administered as a whole body aerosol (Heine et al, 2007). Spores for aerosol challenge were maintained in sterile water and diluted to the challenge dose of ~1×10^{10} CFU/mL. To verify final bacterial concentrations and exposure doses, following serial dilution and plating at 35ºC overnight on sheep blood agar (SBA) plates, colonies were enumerated. The omadacycline and ciprofloxacin MICs against *B. anthracis* Ames were 0.03 mcg/mL.

Y. pestis CO92 was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, which was originally isolated in 1992 from a fatal human case of pneumonic plague. The LD_{50} in mice for this strain was 6.8×10^4 CFU inhaled when administered as a whole body aerosol. The inoculum for aerosol challenge was prepared as previously described (Byrne, 1998) and the suspension of *Y. pestis* was diluted to the appropriate aerosol challenge dose. To verify final bacterial concentrations and exposure doses, colonies were enumerated after serial dilution and plating on SBA plates incubated for 2 days at 28ºC, and colonies were counted. Doxycycline and

ciprofloxacin MICs against *Y. pestis* CO92 were 0.5 µg/mL and 0.06 µg/mL, respectively.

Specific pathogen-free female BALB/c mice (Charles River Laboratories, Frederick, Maryland) weighing approximately 20 g were used throughout the study. Animals were allowed access to food and water *ad libitum* and housed in groups of 10. All procedures were performed in accordance with protocols approved by the USAMRIID Institutional Animal Care and Use Committee, and met or exceeded the standards of the American Association for the Accreditation of Laboratory Animal Care (AAALAC), the United States Department of Health and Human Services, and all local and federal animal welfare laws.

Aerosol Infection

For Study 1, two separate doses of 12.0 and 7.6 times the LD_{50} of *B. anthracis* were administered. For Study 2, four separate doses of 29.8, 27.9, 27.3, and 37.2 times the LD_{50}, for a mean of 30.5 LD_{50} were administered. For *Y. pestis*, mean inhaled doses of 29.4 x LD_{50} (3 separate sprays of 26.1, 32.6, and 30.8 LD_{50}) were administered.

All doses were administered to female BALB/c mice by whole-body aerosol. Challenged mice were then randomized into separate treatment cohorts, balancing challenge doses for each cohort. Aerosols were generated using a three-jet collision nebulizer (May, 1973). All aerosol procedures were controlled

and monitored using the Automated Bio-aerosol Exposure system (Harting, 2004) operating with a whole-body rodent exposure chamber. Integrated air samples were obtained from the chamber during each exposure using an all-glass impinger (AGI). Aerosol bacterial concentrations were serially diluted and plated on SBA plates, as described above. The inhaled dose (CFU/mouse) of *B. anthracis* or *Y. pestis* was estimated using mouse respiratory rates according to Guyton (Guyton, 1947). Mice were randomly placed into separate cages upon the conclusion of each aerosol.

Assessment of Efficacy

For Study 1, omadacycline was administered at doses of 5, 10 or 20 mg/kg given twice daily by intraperitoneal (IP) injection, beginning 24 ± 1 hours after initial *B. anthracis* challenge. The positive comparator was ciprofloxacin 30 mg/kg, administered IP twice daily, starting 24 ± 1 hours after challenge. A second comparator group consisting of doxycycline 10 mg/kg, IP twice daily, was given 24 hours post-challenge to allow comparison of omadacycline to a similar tetracycline class antibiotic. A vehicle control group received 0.1 mL saline IP twice daily. Cohorts consisted of 10 animals.

For Study 2, cohort size was 10 mice, with the exception of only 9 mice in the post-exposure prophylaxis (PEP) ciprofloxacin group. Omadacycline and doxycycline were administered separately at doses of 0.75, 2.5, 7.5 or 15 mg/kg given twice daily by IP injection, beginning 24 ± 1 hours after initial *B. anthracis*

challenge (post-exposure prophylaxis). Additional omadacycline and doxycycline cohorts received 15 mg/kg twice daily beginning 48 ± 1 hours after challenge (delayed treatment). The positive comparator was ciprofloxacin 30 mg/kg, administered IP twice daily, starting 24 ± 1 hours or 48 ± 1 hours after challenge. A vehicle control group received 0.2 mL saline IP twice daily.

Omadacycline and doxycycline were separately administered at doses of 5, 10, 20 or 40 mg/kg given twice daily by IP injection, beginning 24 ± 1 h after initial *Y. pestis* challenge in 10 mice. The positive comparator was ciprofloxacin 15 mg/kg, administered IP twice daily, starting 24 ± 1h after challenge. A vehicle control group received 0.2 ml saline IP twice daily.

Survival was assessed at least twice daily during treatment and at least once daily thereafter. Moribund animals were euthanized as necessary and counted as dead. In accordance with the accepted timeline for these animal models of infection, the study was terminated at 38 to 41 days.

Drug Preparation

Omadacycline was provided as a tosylate salt (1.38 grams of salt form yielded 1 gram of active omadacycline). Omadacycline was prepared in batches for 3 days of use and dosed as 0.1 mL IP injections (~20 g mouse): 20 mg/kg (4 mg/mL) by dissolving 80 mg into 14.5 mL of Phosphate Buffered Saline (PBS); 10 mg/kg (2 mg/mL) by diluting 4 mL of the 4 mg/mL solution with 4 mL of PBS; 5 mg/kg (1

mg/mL) by diluting 2 mL of the 4 mg/ml solution with 6 mL of PBS. A commercially supplied 10 mg/mL stock of ciprofloxacin (Teva Pharmaceutical Industries) was diluted to 6.0 mg/mL with sterile water for injection (SWI) for a 0.1 mL injection (~20 g mouse) dose of approximately 30 mg/kg IP. A commercially supplied 100 mg vial doxycycline (Abraxis BioScience) was resuspended to 10 mg/mL with 10 mL of sterile water for injection, and then further diluted with saline to 2 mg/mL for a 0.1 mL injection dose of approximately 10 mg/kg.

Study Analysis

All analyses were performed employing a stratified Kaplan-Meyer analysis with a log-rank test as implemented on Prism Version 5.04, GraphPad Software.

RESULTS

In Vitro

Omadacycline was active against *B. anthracis* (MIC_{90} = 0.06 mcg/mL) and *Y. pestis* (MIC_{90} = 1 mcg/mL). Omadacycline was less potent than ciprofloxacin against *Y. pestis* (MIC_{90} = 0.03 mcg/mL) but slightly more active against *B. anthracis* (MIC_{90} = 0.12 mcg/mL) (**Table 1**). *In vitro* activity of omadacycline was generally comparable to tetracycline and doxycycline. The distribution of MICs for *B. anthracis* and *Y. pestis* for omadacycline and comparators are shown in Figure 1.

In Vivo

Study 1

The survival curves for all treatment cohorts differed significantly from the vehicle cohort (p=0.004) (**Figure 2**). The median survival for the vehicle-treated controls was 6 days post-challenge. All omadacycline treated mice survived the entire study (38 days) regardless of dose studied (5, 10 and 20 mg/kg twice daily). Additionally, all the ciprofloxacin (30mg/kg twice daily) and doxycycline (10 mg/kg twice daily) -treated mice survived the entire study. Upon study termination, spleens and lungs from surviving mice were excised and the homogenates were plated on SBA plates to determine the degree of *B. anthracis* infection. Consistent with this murine model of inhalational anthrax, residual bacteria (mean ~6.7 CFU/g for treatment cohorts) were recovered from lungs of each surviving mouse. Spleen culture results for all mice were negative, indicating that surviving mice were cleared of bacterial infection. Positive lung results with negative spleens are consistent with the infection model (Heine et al, 2007).

Study 2

In the post-exposure prophylaxis arm, the survival curves for the ciprofloxacin, omadacycline 2.5, 7.5, and 15 mg/kg, and doxycycline cohorts differed significantly (p<0.0001) from the vehicle cohort (**Figure 3**). Furthermore, the doxycycline 2.5, 7.5, and 15 mg/kg cohorts differed significantly (p=0.0354) among each other, but no difference was observed between omadacycline cohorts at the dosages evaluated. The omadacycline 0.75 mg/kg cohort differed significantly (p<0.0004) from vehicle, but no difference was observed for the

matching doxycycline 0.75 mg/kg cohort. Likewise, among the direct comparisons of the four matched omadacycline and doxycycline doses, only the 0.75 mg/kg cohorts differed significantly from each other (p=0.0125). Mean MIC was 0.03 mcg/mL for ciprofloxacin and doxycycline and ≤0.03 mcg/mL for omadacycline. All 10 animals died in the vehicle group with median survival of 2.25 days. Two animals in the 2.5 mg/kg and 6 animals in the 0.75 mg/kg omadacycline groups died, and median survival was 4.75 days in the 0.75 mg/kg group.

The delayed treatment mouse efficacy model for anthrax at USAMRIID has been used with both 42 and 48 hour post-challenge therapeutic initiation; systemic infection is expected anywhere between 42 and 48 hours (Heine et al, 2007). In this arm of the study, the ciprofloxacin 30 mg/kg cohort differed significantly (p=0.0015) from the vehicle cohort, as did the omadacycline 15 mg/kg cohort (p=0.0177) and the doxycycline 15 mg/kg cohort (p=0.0101) (**Figure 4**). No significant differences were observed for the delayed treatment among ciprofloxacin, doxycycline, and omadacycline cohorts. Mean MIC was 0.03 mcg/mL for ciprofloxacin and doxycycline and ≤0.03 mcg/mL for omadacycline. All 10 animals in the vehicle group died with a median survival of 2.25 days. Two, four, and three animals in the ciprofloxacin, omadacycline, and doxycycline groups died.

Y. Pestis In Vivo

Ninety-percent of omadacycline (40mg/kg twice daily) and doxycycline (40 mg/kg twice daily) treated mice survived the entire study (**Figure 5**). All ciprofloxacin-treated mice survived. A dose-response effect for omadacycline and doxycycline was observed, but no significant effect on extended median survival relative to the saline controls was observed for the 5, 10, and 20 mg/kg cohorts. Three representative spleens from each surviving cohort were homogenized and plated for bacteria. Bacterial clearance from the spleens of three ciprofloxacin, three omadacycline (40 mg/kg twice daily), and one doxycycline (40 mg/kg twice daily).

DISCUSSION

Omadacycline demonstrated broad *in vitro* activity against *B. anthracis* and *Y. pestis* isolates. The MIC_{90} values for omadacycline were ≤2 mcg/mL against 100% of the isolates. In general, it is predicted that a MIC of ≤2 mcg/mL supports a potential role of omadacycline for treating anthrax and plague.

In murine infection models for these bacterial agents, the *in vivo* efficacy of omadacycline was also demonstrated. *In vivo* results demonstrated that omadacycline dosed at 5, 10 or 20 mg/kg twice daily for 14 days had significant efficacy over the vehicle control for the treatment of aerosolized *B. anthracis*. Unfortunately, 4 of 10 vehicle-treated mice survived the relative low challenge of *B. anthracis* spores. While there was a statistically significant treatment effect of omadacycline suggesting that omadacycline might be an effective post-exposure prophylaxis option for treating anthrax, the lack of mortality in the untreated

controls resulted in studying anthrax in a delayed treatment exposure model. The delayed treatment exposure revealed efficacy with omadacycline that was comparable to ciprofloxacin and doxycycline. The delayed treatment exposure is a more robust evaluation of the efficacy of treatments in this model.

Additionally, results from the higher inoculum *B. anthracis* study demonstrated that when dosed at 2.5, 7.5, and 15 mg/kg and as low as 0.75 mg/kg twice daily for 14 days, omadacycline had significant efficacy over the vehicle control in the treatment of aerosolized *B. anthracis*. A similar finding was observed with doxycycline, but only at doses as low as 2.5 mg/kg twice daily for 14 days. When dosed sufficiently, both doxycycline and omadacycline were effective as post-exposure prophylaxis against inhalational anthrax in this model, and both were comparable to ciprofloxacin. Both doxycycline and omadacycline also performed well and were comparable to ciprofloxacin for delayed treatment experiments with *B. anthracis*.

Similarly, when dosed at 40 mg/kg twice daily for 7 days, both omadacycline and doxycycline demonstrated significant efficacy for *Y. pestis*. When dosed sufficiently, both doxycycline and omadacycline were as effective as ciprofloxacin in this model. At 20 mg/kg twice daily, omadacycline significantly outperforms doxycycline but is nonetheless ineffective as PEP against inhalational plague.

While sporadic cases of plague are reported yearly worldwide, a rapid spread of this pathogen as part of a bioterrorism act could have devastating effects on the population (U.S. Labor Department). Inhalational anthrax carries with it the most serious complications of biothreat agents and a mortality rate of 90% or more (Jernigan et al, 2002; Konig, 2013; Holty et al, 2006; Sweeney et al, 2011; Goel, 2015). While anthrax and plague are generally susceptible to tetracylines as well as other widely available antibiotic classes, incidents of resistance have been reported.

Based on its *in vitro* activity, its well-characterized pharmacokinetics after oral and IV administration, and its safety and tolerability profile, omadacycline offers an attractive treatment option for some of the more serious biothreat organisms evaluated in these studies. As a novel aminomethylcycline compound that has been engineered to overcome existing tetracycline resistance mechanisms of efflux and ribosomal protection, omadacycline might offer a viable treatment alternative where current therapies are not indicated due to host drug reactions or bacterial resistance. This strongly indicates that evaluation in the other infection models should be considered.

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Table 1. MIC (mcg/mL) values for 30 strains of *B. anthracis* and *Y. pestis* for omadacycline vs. comparators.

B. anthracis (30 strains)	Omadacycline	Ciprofloxacin	Tetracycline	Doxycycline
MIC range	<0.03 – 0.06	0.03 – 0.25	<0.03 – 1	0.03 – 0.06
MIC_{50}	0.03	0.06	<0.03	0.03
MIC_{90}	0.06	0.12	0.12	0.06
Y. pestis (30 strains)				
MIC range	0.12 – 2	0.004 – 0.06	0.25 – 2	0.06 – 2
MIC_{50}	1	0.015	0.5	0.5
MIC_{90}	1	0.03	2	1

Figure 1. Distribution of MICs (n=30 strains) for omadacycline and comparators for *B. anthracis* and *Y. pestis*.

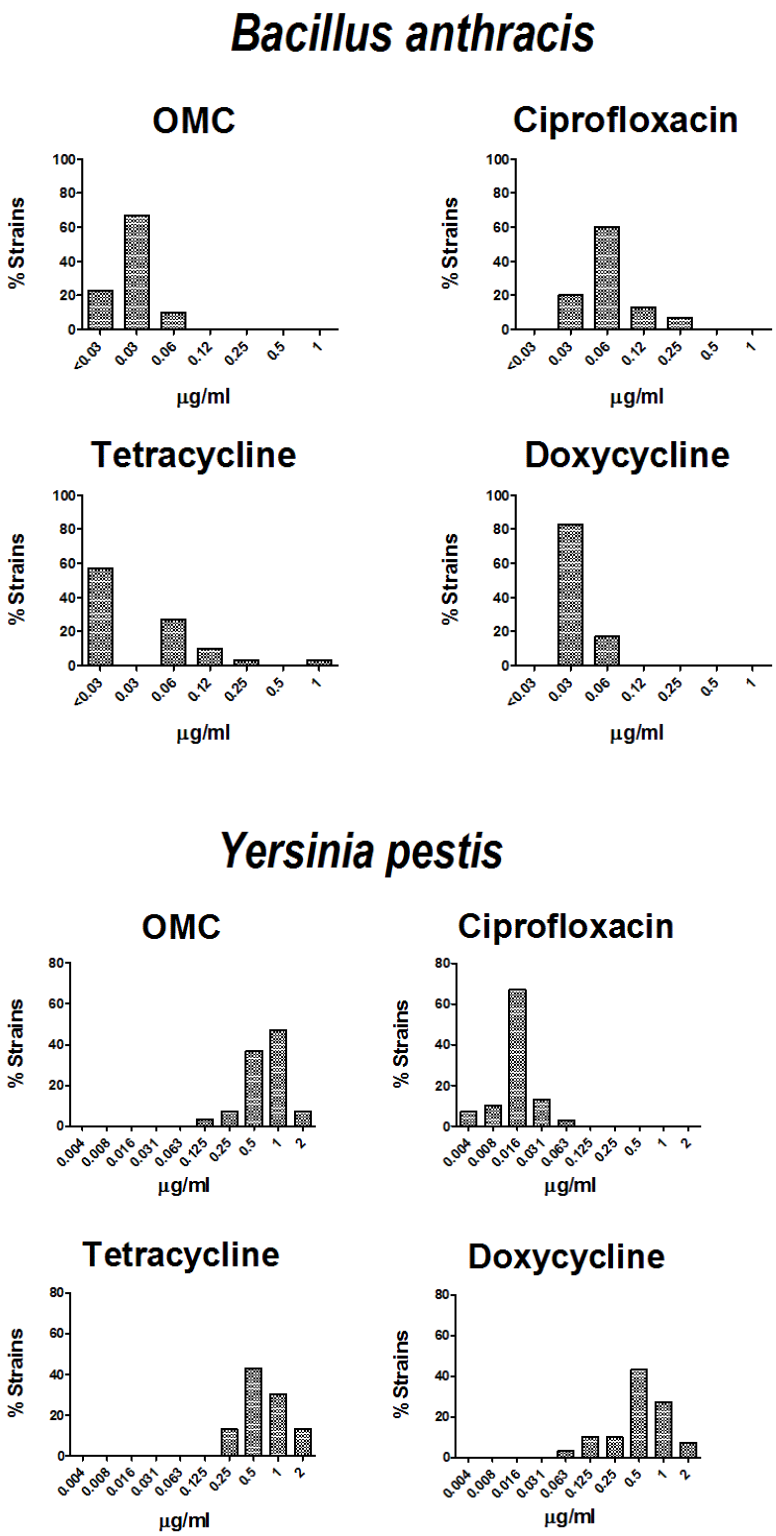

Figure 2. Study 1: Survival of mice infected with *B. anthracis* Ames following treatment with omadacycline, doxycycline or ciprofloxacin (all IP): all groups N=10. Post-exposure prophylaxis.

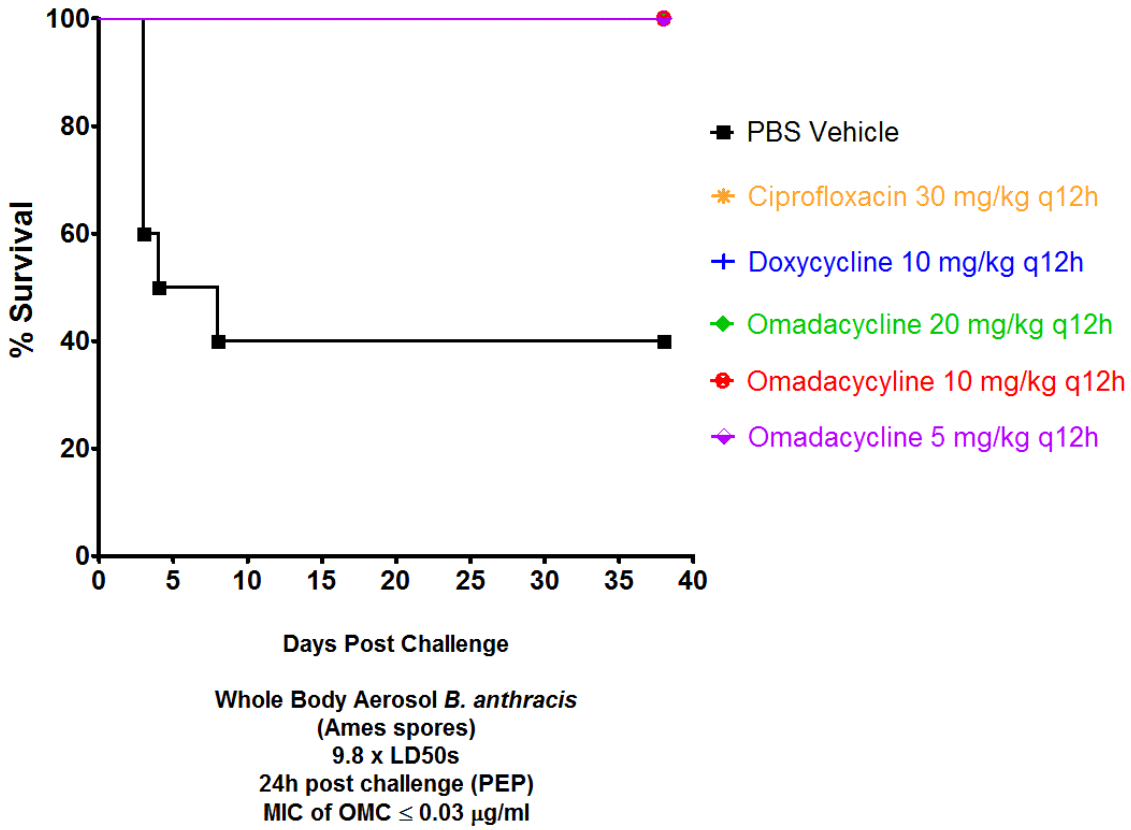

Figure 3. Study 2: Post-exposure prophylaxis. Survival of mice infected with *B. anthracis* Ames following treatment with omadacycline, doxycycline or ciprofloxacin (all IP): All Groups (N=10) [N=9 Ciprofloxacin]. MIC for ciprofloxacin and doxycycline = 0.03 mcg/mL and for omadacycline ≤0.03 mcg/mL.

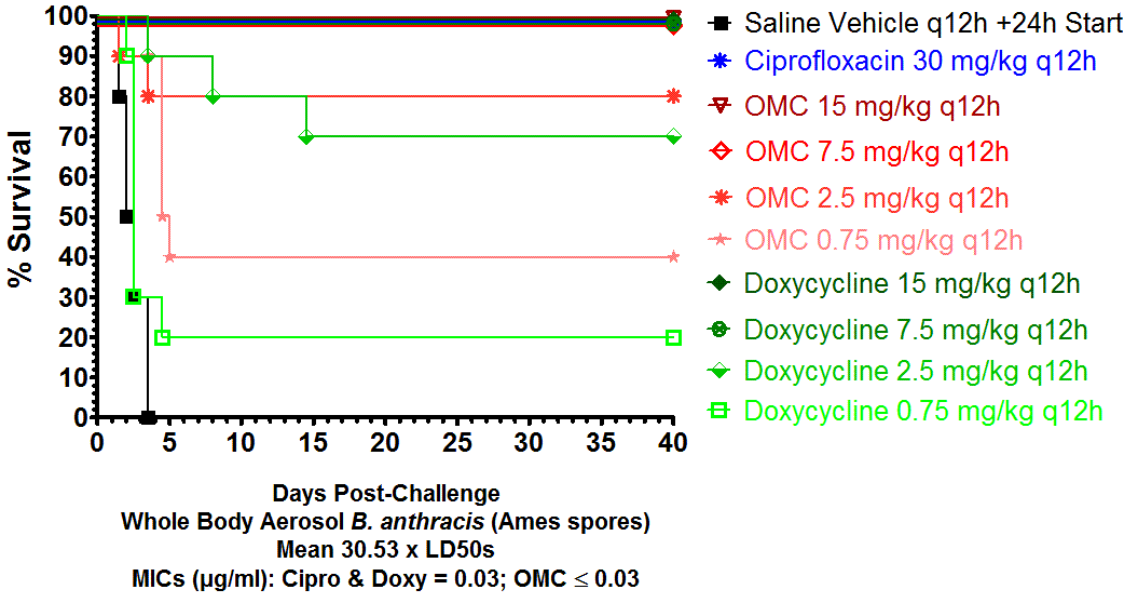

Figure 4. Study 2: Delayed Treatment, 48 h after treatment initiation. Survival of mice infected with *B. anthracis* Ames following treatment with omadacycline, doxycycline or ciprofloxacin (all IP): All Groups (N=10) [N=9 Ciprofloxacin]. MIC for ciprofloxacin and doxycycline = 0.03 mcg/mL and for omadacycline ≤0.03 mcg/mL.

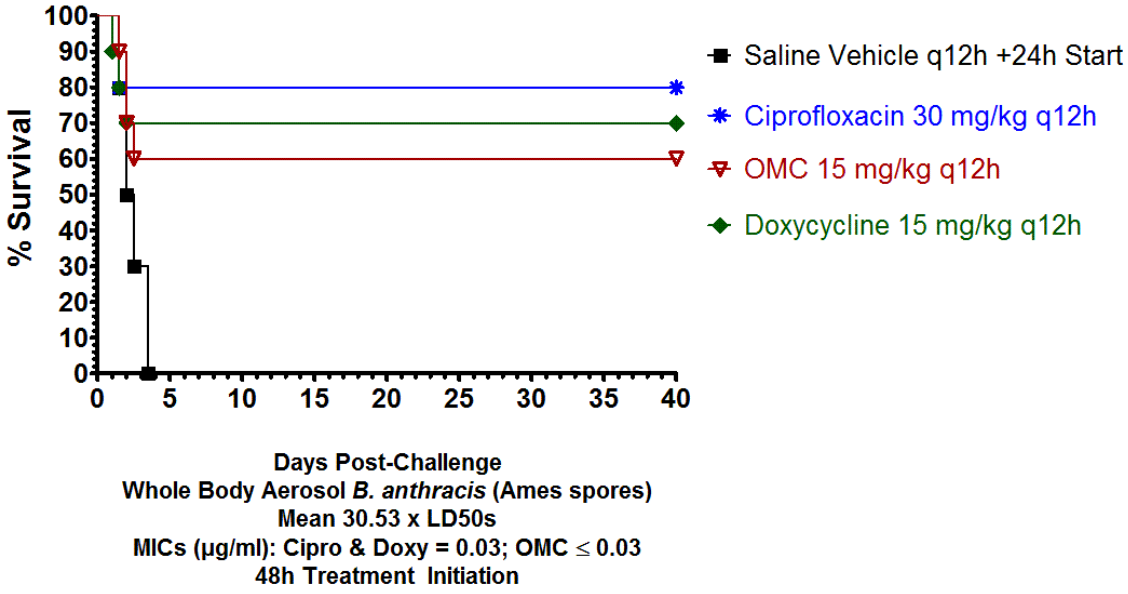

Figure 5. Post-exposure prophylaxis. Survival of mice infected with *Y. pestis* following treatment with omadacycline, doxycycline or ciprofloxacin (all IP): all groups N=10. MIC for doxycycline = 0.5 mcg/mL and for omadacycline 1 mcg/mL.